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### Citation for published version:

Fitch, PM, Wheelhouse, NM, Bowles, P, Paterson, M, Longbottom, D, Entrican, G & Howie, SEM 2010, 'Ectopic lymphoid tissue formation in the lungs of mice infected with *Chlamydia pneumoniae* is associated with epithelial macrophage inflammatory protein-2/CXCL2 expression', *Clinical & Experimental Immunology*, vol. 162, no. 2, pp. 372-378. <https://doi.org/10.1111/j.1365-2249.2010.04231.x>

### Digital Object Identifier (DOI):

[10.1111/j.1365-2249.2010.04231.x](https://doi.org/10.1111/j.1365-2249.2010.04231.x)

### Link:

[Link to publication record in Edinburgh Research Explorer](#)

### Document Version:

Publisher's PDF, also known as Version of record

### Published In:

Clinical & Experimental Immunology

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# Ectopic lymphoid tissue formation in the lungs of mice infected with *Chlamydia pneumoniae* is associated with epithelial macrophage inflammatory protein-2/CXCL2 expression

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Accepted for publication 24 June 2010

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## Summary

Infection with *Chlamydia pneumoniae* (Cp) accounts for around 10% of community acquired bacterial pneumonia and has been associated with other chronic inflammatory conditions. We describe a C57/Bl6 murine model of Cp lung infection characterized by a dose-dependent, resolving neutrophilia followed by lymphocytic infiltration of the lungs. By 21 days post-infection, mice exhibit a T helper type 1 (Th1) polarized serum antibody response with local mucosal antibody secretion and organization of ectopic lymphoid tissue which persisted in the absence of detectable Cp DNA. Macrophage inflammatory protein (MIP)-2/CXCL2, which recruits neutrophils and lymphocytes and is associated with ectopic lymphoid tissue formation, was secreted in the lungs post-infection. *In vitro*, lung epithelial cells up-regulated MIP-2/CXCL2 in response to both rough lipopolysaccharide (reLPS) and Cp infection. We conclude that Cp infection can have long-term inflammatory effects on tissue that persist after clearance of active infection.

**Keywords:** *Chlamydia pneumoniae*, CXCL2/MIP-2, epithelium, lung, lymphocytes

## Introduction

*Chlamydia pneumoniae* (Cp), an obligate intracellular bacterium, is a ubiquitous human pathogen [1]. Cp infection accounts for around 10% of community acquired bacterial pneumonia and has been associated with chronic lung diseases including chronic bronchitis [2], asthma [1] and chronic obstructive pulmonary disease (COPD) [3] and other chronic pathologies, including multiple sclerosis [4], atherosclerosis [5] and Alzheimer's disease [6]. Despite these associations, antibiotic therapy does not have a major clinical benefit in these disorders [7–9], and where an effect has been observed, such as with macrolides [10,11] it is unclear if this is due to anti-microbial or anti-inflammatory effects [12]. It is possible that pulmonary Cp infection initiates an inflammatory environment which persists after bacterial clearance and contributes to infection-associated pathology.

Several murine models of Cp 'vaccination', infection and reinfection have been published [13–15] and have established that Cp infects lung epithelial cells and induces lung inflammation, ectopic lymphoid tissue formation and airway hyper-reactivity. However, there is little information on how resolution of infection relates to inflammation-induced disruption of lung architecture which may contribute to pathology.

We investigated a link between cytokine production by Cp-infected lung epithelial cells and inflammation. The chemokine macrophage inflammatory protein (MIP)-2/CXCL2 recruits both neutrophils and lymphocytes [16–18] and is associated with ectopic lymphoid tissue formation [19]. It has been reported that epithelial cell secretion of MIP-2/CXCL2 recruits both lymphocytes and neutrophils to the gut [18]. Here we report that murine Cp lung infection induced systemic T helper type 1 (Th1)-driven immunity, local mucosal antibody secretion and initiated organization of ectopic lymphoid tissue which persisted in the absence of detectable Cp DNA. We demonstrated that MIP-2/CXCL2 was secreted in the lungs post-infection (PI). Furthermore, *in vitro* studies utilizing lung epithelial cell lines demonstrated up-regulation of MIP-2/CXCL2 in response to both Cp infection and to a rough form of LPS (reLPS) analogous to that expressed by Cp.

## Materials and methods

### Reagents and plastics

Unless stated, plastics were from Costar (Fisher Scientific, Loughborough, UK), culture reagents from Invitrogen (Paisley, UK) and other reagents from Sigma (Poole, UK).

## Bacteria

HEp2 cell (ECACC, Salisbury, UK) monolayers were washed and incubated with diethylaminoethyl (DEAE)-dextran 30 µg/ml in Hanks' balanced salt solution (HBSS) (20 min, room temperature). DEAE-dextran/HBSS was removed and Cp AR39 strain (ATCC 53592) in infection medium [5% fetal calf serum (FCS)/Iscove's modified Dulbecco's medium (IMDM)/1 µg/ml cycloheximide] added for 3 h and then replaced with fresh infection medium alone. Infected monolayers were disrupted manually with glass beads. The suspension was centrifuged at 200 g/10 min/4°C, the supernatant centrifuged further at 22 000 g/40 min/4°C, the resulting pellet resuspended in sucrose-phosphate-glutamate buffer [20] medium and stored at -80°C. Uninfected HEp2 lysate was used as a control. Stocks were titrated in Hep2 cells as described for *C. abortus* [21]. Titres of stocks were  $7 \times 10^7$ – $10^8$  inclusion-forming units (IFU)/ml. Cp were inactivated by exposure to 2 mJ/cm<sup>2</sup> under an ultraviolet (UV)-cross-linker.

## In vivo infection

Six to 8-week-old C57Bl/6 mice, bred and maintained under standard specific pathogen-free conditions, were used with local ethical and UK Home Office approval. Fifty µl phosphate-buffered saline (PBS), containing live or UV-inactivated Cp, or control lysate, was instilled into the trachea under anaesthetic. Mice were killed at various times PI. As described [22], blood was taken; bronchoalveolar lavage fluid (BALF) collected; lungs were perfused with PBS via the heart, removed, inflated with and placed into methacarn fixative overnight before processing and wax embedding. Serum was aliquoted and stored at -20°C until use. For studies involving Cp DNA detection, lungs were processed as above, with the exception that the single left lobe was tied off and excised for DNA extraction prior to inflation of the right lobes with fixative for immunohistochemistry and histology.

## Cp DNA detection

DNA was extracted (Wizard genomic DNA purification kits; Promega, Southampton, UK) from homogenized lung lobes (TissueLyser, Qiagen, Crawley, UK) and run on an Applied Biosystems (Warrington, UK) 7500 real-time polymerase chain reaction (PCR) machine as duplicate single reactions using Applied Biosystems mastermix for host 18S primers and Cp 23S primers and probes [23]. Ct values were used to generate a relative ratio between DNA for bacterial 23S rRNA and host 18S rRNA.

## Inflammation in BALF

BALF was centrifuged at 300 g/5 min/4°C, supernatant removed and stored at -20°C. Cells were counted, cytopins

spun (Shandon cytocentrifuge; Fisher Scientific), methanol-fixed, stained with DiffQuik (CellPath Store, Powys, UK) and differential cell counts performed by an observer blinded to experimental details (S. E. M. H.).

## Immunohistochemistry (IHC)

Three-µm sections were dewaxed, rehydrated, blocked with 3% H<sub>2</sub>O<sub>2</sub> in methanol (5 min, room temperature) and antigen retrieved (Borg decloaker; Biocare Medical, Concord, CA, USA). For IHC with mouse monoclonal antibody, Vector Laboratories' (Peterborough, UK) mouse-on-mouse kit was used. Slides in Shandon Sequenza™ staining clips (Fisher Scientific) were washed, primary antibody (1:100 goat anti-murine MIP-2/CXCL2 antibody, R&D Systems, Abingdon, UK; 1:50 rat anti-mouse IgA, 1:125 rat anti-mouse B220, 1:300 rat anti-mouse CD138, all BD Biosciences, Oxford, UK; 1:200 rabbit anti-CD3 antibody, Dako, Ely, UK; 1:1000 mouse monoclonal anti-LPS antibody, clone 13/4, in-house [24]) or diluent control added (overnight 4°C), washed, 1:250 biotinylated secondary antibody (swine anti-rabbit IgG, rabbit anti-goat IgG, rabbit anti-mouse IgG, all Dako; goat anti-rat IgG, Vector Laboratories) added (1 h, room temperature), washed, streptavidin-horseradish peroxidase (HRP) (Dako) added, washed, and staining visualized with diaminobenzidine substrate solution (Dako).

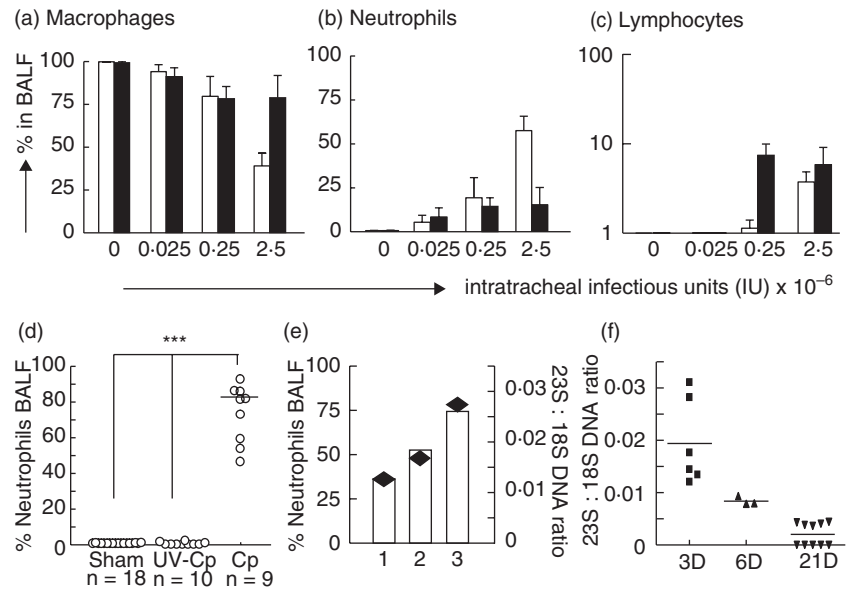
## Assessment of lung inflammation

As described [22], inflammation was scored by an observer blinded to experimental details (S. E. M. H.) in perivascular and peribronchiolar tissue (1 = 0, 2 = < 20, 3 = > 20 < 100 and 4 = > 100 infiltrating cells) on haematoxylin and eosin (H&E)-stained 3-µm sections at ×200 magnification by averaging the score of 10 consecutive fields where the lungs were inflated correctly and the field contained a complete transection of at least one bronchiole (< 300 µm diameter). Neutrophilia was determined as percentage of infiltrating cells.

## Enzyme-linked immunosorbent assay (ELISA) for rough LPS antibodies

Antibodies to Cp rough LPS (reLPS) are cross-reactive with *Salmonella minnesota* reLPS [25], which was used to measure antibody responses to infection. Ninety-six-well ELISA plates were coated with 100 ng/well reLPS in pH9.6 carbonate/bicarbonate buffer (overnight, 4°C), washed, blocked (1 h, room temperature) with 1% bovine serum albumin (BSA), washed, serum or BALF dilutions added (overnight, 4°C), washed, biotinylated anti-mouse immunoglobulin IgG1 (Serotec, Oxford, UK), biotinylated anti-mouse IgG2a<sup>b</sup> or biotinylated anti-mouse IgA (both BD Biosciences) added (1 h, room temperature), washed, streptavidin-HRP (R&D Systems) added (1 h, room temperature), washed and substrate (hydrogen peroxide/

**Fig. 1.** Bronchoalveolar fluid (BALF) macrophages (a), neutrophils (b) and lymphocytes (c) 3 days (clear bars) and 6 days (black bars) post-infection (PI). (d) Neutrophilia in BALF 3 days PI with  $2.5 \times 10^6$  live, but not ultraviolet (UV)-inactivated, international units (IU) Cp. \*\*\* $P < 0.001$  compared to UV-inactivated *Chlamydia pneumoniae* (Cp) and to sham. (e) Degree of neutrophilia (white bars) in three representative mice 3 days PI with  $2.5 \times 10^6$  IU correlates with detection of Cp DNA in lung tissue (black diamonds). (f) Detection of Cp DNA in lung tissue at various times PI.



tetramethylbenzidine; R&D Systems) added (30 min, 37°C, in the dark). The reaction was stopped by addition of 2 N  $H_2SO_4$  and plates read at 450 nm (650 nm reference) on a Biotek Synergy HT plate reader (Fisher Scientific).

#### *In vitro* LPS treatment and infection

Cells were cultured in a humidified incubator at 37°C in 5%  $CO_2$ . Mycoplasma-free, C57Bl/6 derived pulmonary epithelial cell lines, mtCC1-2 [26] and CMT64/61 (ECACC) were maintained in antibiotic-free IMDM containing 10% FCS and 1% L-glutamine (complete IMDM); 250  $\mu$ l complete IMDM, containing  $2.5 \times 10^5$  cells, was plated into 48-well plates, cultured for 24 h, medium removed, fresh medium containing reLPS (10  $\mu$ g/ml), or Cp (at MOI 10), or control lysate, added to triplicate wells and cultured for various times.

#### MIP-2/CXCL2-2 protein and m-RNA detection

MIP-2/CXCL2 protein was detected using R&D Systems murine DuoSet ELISA. RNA (extracted using RNeasy micro-kit; Qiagen) was run in triplicate multiplex reactions (25  $\mu$ l containing TaqMan reagents and 40 ng cDNA) in an Applied Biosystems 7500 real-time PCR machine. Gene expression normalized to 18SRNA was linearized to give fold change relative to a value of 1 assigned to expression in cultures grown in medium alone.

#### Statistics

Results were analysed by unpaired *t*-test with Welch's correction using Graphpad Prism™ software.

## Results

### *In vivo* infection

Dose-dependent BALF neutrophilia was resolving by day 6 PI, whereas the % of lymphocytes, although small, increased from day 3 to day 6 (Fig. 1a–c). Subsequently a dose of  $2.5 \times 10^6$  IU was used; this induced severe neutrophilia which was not seen with UV-inactivated Cp (Fig. 1d). BALF neutrophilia 3 days PI correlated with chlamydial 23S DNA in lungs from the same animals (Fig. 1e). Bacterial DNA declined after infection and by 21 days PI was undetectable in five of 10 animals (Fig. 1f).

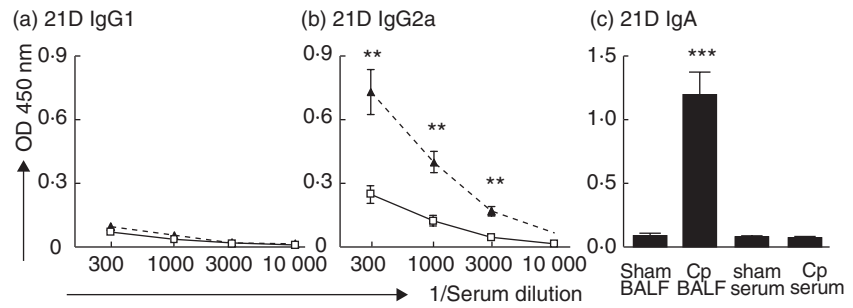
### Cp lung infection induces systemic IgG2a and local IgA antibody

Twenty-one days PI, high-titre anti-reLPS IgG2a, but not IgG1, antibodies were detected in serum from infected mice (Fig. 2a,b). There were no detectable anti-reLPS serum IgA antibodies, but high levels were seen in BALF from infected mice (Fig. 2c).

### Kinetics of the inflammatory response to Cp infection in lung tissue

Sham-infected mice showed no inflammation at any time, so sham data have been pooled. Figure 3a–g shows inflammation in BALF where total cells and neutrophilia peaked 3 days PI and returned to normal by 21 days PI whereas lymphocytes remained raised 21 days PI. The macrophage number did not differ from the sham-infected controls. Levels of the macrophage chemokine MCP-1 did not differ between BALF from infected or uninfected animals (not shown).

**Fig. 2.** Anti-rough lipopolysaccharide (reLPS) antibodies 21 days post-infection (PI). Immunoglobulin (Ig)G1 (a) and IgG2a antibodies (b) in serum [dotted lines *Chlamydia pneumoniae* (Cp)-infected, solid lines sham-infected mice]. (c) Bronchoalveolar fluid (BALF) and serum (all at 1:30 dilution) IgA antibodies. Sham,  $n = 8$ ; Cp-infected,  $n = 10$ ; \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$  compared to sham controls.



Inflammation in lung interstitium (Fig. 3h–j) also showed neutrophilia that peaked on day 3 and resolved by day 21 PI. In contrast, perivascular and peribronchiolar inflammation did not resolve by day 21 PI.

### Cp infection induces formation of ectopic lymphoid tissue

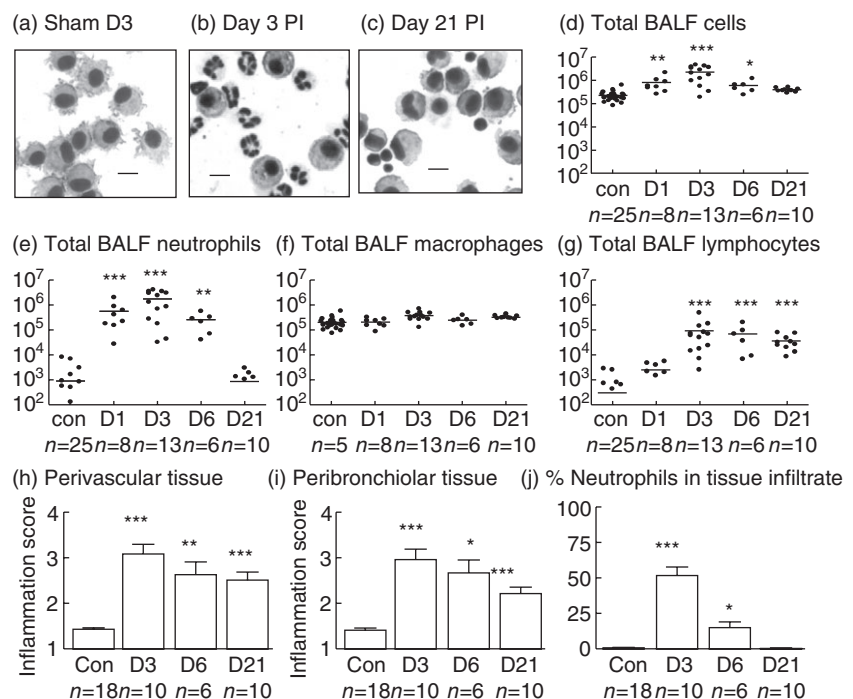
Lungs from mice 21 days PI showed multiple, patchy lymphocytic aggregates. The percentage of blood vessels (mean number/section,  $78 \pm 7$ ) and bronchioles (mean number/section,  $34 \pm 4$ ) associated with  $> 10$  T lymphocytes (on anti-CD3 stained sections) and with  $> 10$  B lymphocytes (on anti-B220 stained sections) were determined. The percentage of blood vessels or bronchioles associated with either cell type was  $< 1$  for sham-infected mice, whereas 30–40% of blood vessels and 10–20% of bronchioles in infected mice had associated lymphoid aggregates (Fig. 4a,b). Many aggregates were large, morphologically resembled germinal

centres (Fig. 4c,d) and contained clusters of B lymphocytes (Fig. 4e) and scattered T lymphocytes (Fig. 4f). Large numbers of cells were positive for IgA (Fig. 4g) and staining for CD138 (a marker of antibody secreting plasma cells and of lung bronchiolar epithelial cells [27,28]) revealed positive plasma cells indicating ectopic lymphoid tissue formation (Fig. 4h).

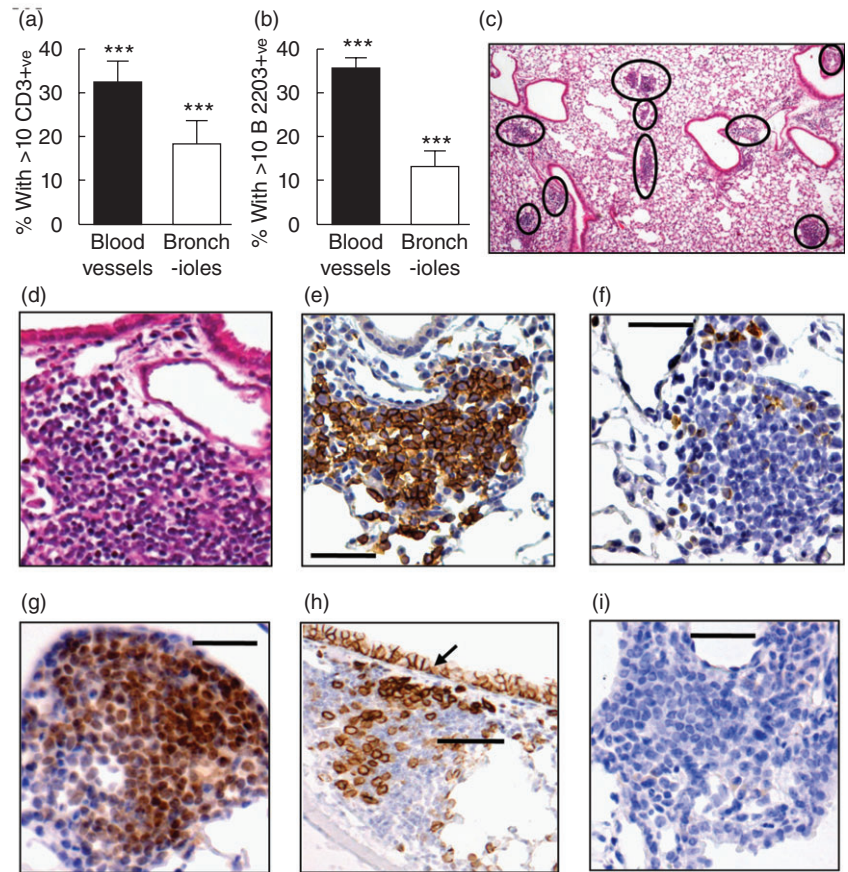
### Lymphoid aggregates, anti-reLPS antibodies and lymphocytes in BAL do not correlate with detection of 23S chlamydial DNA

Twenty-one days PI, approximately half the mice had no detectable chlamydial 23S DNA in the lungs. There were no differences between IgA in BALF, IgG2a in serum (Fig. 5a), total BALF lymphocytes (Fig. 5b) or distribution of lymphoid aggregates (Fig. 5c,d) in mice that were positive or negative for detection of chlamydial 23S DNA in the lungs 21 days PI.

**Fig. 3.** Kinetics of lung inflammation. (a–c) representative bronchoalveolar fluid (BALF) cytopins, (a) 3 days after sham infection; (b) 3 days post-infection (PI); (c) 21 days PI; bar = 15  $\mu$ m. (d–g) cells in BALF 1, 3, 6 and 21 days PI compared to pooled sham-infected mice. The majority of sham-infected animals had no detectable lymphocytes or neutrophils in BAL; (d) total cells; (e) total neutrophils; (f) total macrophages; (g) total lymphocytes; (h) perivascular and (i) peribronchiolar lung inflammation scores; (j) neutrophilia in pulmonary tissue. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  compared to sham-infected. Pooled data from four experiments.







**Fig. 4.** Distribution of T (a) and B lymphocytes (b) around pulmonary blood vessels and bronchioles 21 days post-infection (PI); sham-infected mice < 1% for all values. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , compared to sham-infected, pooled data from four experiments. (c) Representative low-power haematoxylin and eosin (H&E), lymphoid aggregates circled. (d) High-power H&E of a single aggregate. (e–i) Representative immunohistochemistry of lung aggregates stained with (e) anti-B220; (f) anti-CD3; (g) anti-immunoglobulin (Ig)A; (h) anti-CD138 (which is also expressed by lung epithelial cells, arrowed); (i) no-primary antibody control. Bars = 100  $\mu$ m.

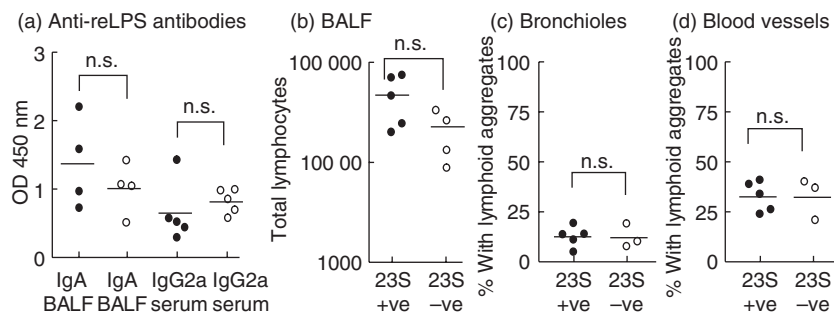
### Infected lungs and airway epithelial cells secrete MIP-2/CXCL2 in response to reLPS and Cp infection

The principal site of infection detected by anti-LPS staining was bronchial epithelial cells (BECs) (Fig. 6a). At 3 days PI, both BECs and alveolar cells contained MIP-2/CXCL2 (Fig. 6b) but at 21 days PI only BECs were positive (Fig. 6c). MIP-2/CXCL2 protein was detected in BALF 1, 3 and 6 days PI (Fig. 6d).

*In vitro*, reLPS induced upregulation of MIP-2/CXCL2 mRNA (Fig. 6e) and protein secretion (Fig. 6f) in two different mouse lung epithelial cell lines. Protein secretion also increased in response to Cp infection (Fig. 6g).

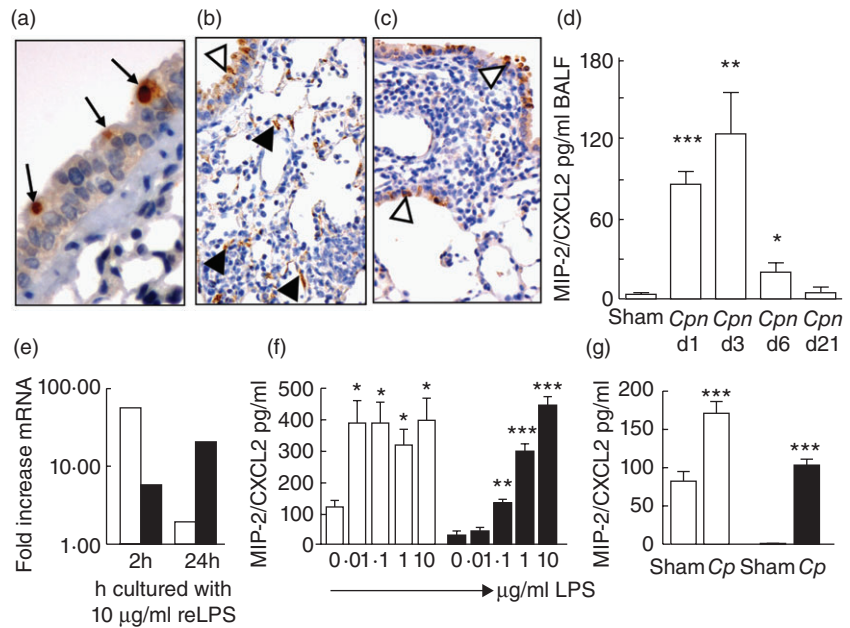
### Discussion

Pulmonary Cp infection induced a dose-dependent, resolving neutrophilia followed by a lymphocytic lung infiltration.



**Fig. 5.** 23S DNA-positive (black circles) and -negative (open circles) lungs. (a) Bronchoalveolar fluid (BALF) immunoglobulin (Ig)A (1:30 dilution) and serum IgG2a (1:300 dilution) anti-rough lipopolysaccharide (reLPS) antibody enzyme-linked immunosorbent assay. (b) Total number of lymphocytes in BALF. (c) Percentage of bronchioles associated with lymphoid aggregates. (d) Percentage of lung blood vessels associated with lymphoid aggregates. Each circle represents an individual mouse.

**Fig. 6.** (a) Lipopolysaccharide (LPS)-positive inclusion bodies in bronchial epithelial cells (BECs) (arrowed), 3 days post-infection (PI); (b) macrophage inflammatory protein (MIP)-2/CXCL2 in BECs (black arrowheads) and alveolar lung cells (white arrowheads) 3 days PI; (c) MIP-2/CXCL2 in BECs (asterisk) 21 days PI; (d) MIP-2/CXCL2 protein in bronchoalveolar fluid (BALF); (e–g) pooled data from four experiments. MIP-2/CXCL2 in epithelial cell lines (white bars, mtCC1-2; black bars, CMT64/61); 10 µg/ml rough LPS (reLPS) up-regulated MIP-2/CXCL2 mRNA (e) and MIP-2/CXCL2 protein secretion (f) after 24 h culture. (g) *Chlamydia pneumoniae* (Cp) infection (MOI 10) up-regulated MIP-2/CXCL2 secretion by epithelial cell lines after 24 h culture. \*\*\**P* < 0.001; \*\**P* < 0.01; \**P* < 0.05 compared to control.



Macrophages in BALF did not change, indicating that macrophage recruitment may not play a significant role in Cp clearance. Alternatively, Cp may have developed defence mechanisms to ensure that macrophages are not recruited by the inflammatory process that follows lung epithelial cell infection. The lack of local MCP-1 up-regulation by Cp infection may support the latter hypothesis. Failure to recruit macrophages during neutrophilia could result in delayed clearance of apoptotic neutrophils, impacting negatively upon the resolution of inflammation. Cp infection induced serum IgG2a and lung IgA antibodies to reLPS, indicating that the systemic antibody response was Th1-driven and that there was a local mucosal response.

Ectopic lymphoid tissue is a feature of chronic inflammation [29]. In this model Cp lung infection favoured development of ectopic lymphoid tissue which secreted IgA locally in the absence of detectable bacterial 23S DNA. Although we cannot exclude a level of infection below the sensitivity of the PCR-based detection method used, it may be that the lymphoid tissue is maintained by an inflammatory process that once initiated does not require active infection. Ectopic lymphoid tissue formation has been reported by others after reinfection of mice with Cp [13,14]. Here we explored a role for infected lung epithelium in this process. Various signals are necessary for ectopic lymphoid tissue formation in inflammatory settings [30], but the initiating mechanisms are not understood completely. MIP-2/CXCL2 is important for recruitment of neutrophils and lymphocytes by gut epithelial cells [18]; in lymphocyte trafficking during autoimmune responses [16]; and for recruitment of lymphocytes to peripheral organs in graft-versus-host disease [17]; and has been implicated in the neo-angiogenesis associated with ectopic lymphoid tissue formation [19]. The finding that Cp infection induces lung epithelial cell expression of MIP-2/

CXCL2 and secretion both *in vivo* and *in vitro* suggests a mechanism by which both neutrophilia and lymphocytosis may develop and formation of ectopic lymphocyte tissue may be initiated by Cp infection of lung epithelial cells *in vivo*. Ectopic lymphoid tissue which disrupts the lung architecture may itself cause signs of disease. Thus MIP-2/CXCL2 induced by Cp infection may initiate lymphocytic lung infiltration that can have long-term effects on tissue after clearance of active infection.

## Disclosure

None of the authors has any conflict of interest to disclose.

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